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Thank-you!

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Genetic engineering of plants to enhance resistance to fungal pathogens—a review of progress and future prospects

Zamir K. Punja

Abstract: Recent applications of techniques in plant molecular biology and biotechnology to the study of host–pathogen interactions have resulted in the identification and cloning of numerous genes involved in the defense responses of plants following pathogen infection. These include: genes that express proteins, peptides, or antimicrobial compounds that are directly toxic to pathogens or that reduce their growth in situ; gene products that directly inhibit pathogen virulence products or enhance plant structural defense genes, that directly or indirectly activate general plant defense responses; and resistance genes involved in the hypersensitive response and in the interactions with avirulence factors. The introduction and expression of these genes, as well as of antimicrobial genes from nonplant sources, in a range of transgenic plant species have shown that the development of fungal pathogens can be significantly reduced. The extent of disease reduction varies with the strategy employed as well as with the characteristics of the fungal pathogen, and disease control has never been complete. Manipulation of salicylic acid, ethylene, and cytokinin levels in transgenic plants have provided some interesting results with regard to enhanced disease tolerance or susceptibility. The complex interactions among the expressed gene product, plant species, and fungal pathogen indicate that the response of transgenic plants cannot be readily predicted. Combinations of defense gene products have shown considerably more promise in reducing disease than single-transgene introductions. The use of tissue-specific or pathogen-inducible promoters, and the engineered expression of resistance genes, synthetic antimicrobial peptides, and elicitor molecules that induce defense responses have the potential to provide commercially useful broad-spectrum disease resistance in the not-too-distant future. The issues and challenges that will need to be addressed prior to the widespread utilization of these transgenic plants are highlighted.

Key words: antifungal proteins, antimicrobial peptides, biotechnology, elicitors, hypersensitive response, pathogenesis-related proteins, phytoalexins, resistance genes, transgenic plants.

Résumé : Les application récentes au domaine végétal des techniques de la biologie moléculaire et de la biotechnologie pour l'étude des interactions hôtes–pathogènes ont permis d'identifier et de cloner plusieurs gènes impliqués dans les réponses de défense des plantes par suite d'une infection par un agent pathogène. Ceux-ci comprennent : des gènes qui codent pour des protéines, des peptides ou des composés antimicrobiens qui ont une toxicité directe ou réduisent la croissance in situ des agents pathogènes; des produits géniques qui inhibent directement des produits de virulence des agents pathogènes ou stimulent les gènes de défense structurale des plantes, qui activent directement ou indirectement les réponses générales de défense des plantes et des gènes de résistance impliqués dans la réaction d'hypersensibilité et dans les interactions avec les facteurs d'avirulence. L'introduction et l'expression de ces gènes, ainsi que celles de gènes antimicrobiens de source non végétale, dans une gamme d'espèces végétales transgéniques ont montré que le développement des champignons pathogènes peut être significativement réduit. L'ampleur de la réduction de la maladie dépends de la stratégie utilisée aussi bien que des caractéristiques du champignon pathogène; une répression complète de la maladie n'a pas encore été atteinte. La manipulation des niveaux d'acide salicylique, d'éthylène et de cytokinines dans des plantes transgéniques a fourni quelques résultats intéressants concernant la tolérance ou la sensibilité accrues à la maladie. Les interactions complexes entre les produits géniques exprimés, les espèces végétales et les champignons pathogènes laissent voir que la réponse des plantes transgéniques ne peut être parfaitement prédite. Les combinaisons de produits géniques de défense ont été beaucoup plus prometteuses

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que les introductions d'un seul transgène pour lutter contre les maladies. L'emploi de promoteurs spécifiques à un tissu ou inductibles par les agents pathogènes et le génie appliqué à l'expression de gènes de résistance, de peptides synthétiques antimicrobiens et de molécules inductrices qui stimulent les réponses de défense ont le potentiel pour fournir commercialement, dans un futur pas si lointain, un moyen pratique pour résister à un large spectre de maladies. Les problèmes et les défis qui devront être surmontés avant d'arriver à une utilisation à grande échelle de ces plantes transgéniques sont mis en évidence.

Mots clés : protéines antifongiques, peptides antimicrobiens, biotechnologie, éliciteurs, réaction d'hypersensibilité, protéines reliées à la pathogenèse, phytoalexines, gènes de résistance, plantes transgéniques.

Introduction

One of the challenges facing breeders during the development of improved crop cultivars for agricultural use is the incorporation of resistance to diseases. Since domestication of plants for human use began, diseases have caused major yield losses and have impacted the well-being of humans worldwide (Agrios 1997). The incorporation of disease resistance genes into plants has been successfully achieved using conventional breeding methods, which involve selection and evaluation of large progeny populations derived from crosses made between resistant and susceptible parents and subsequent screening under disease conducive conditions. Virtually all agricultural crop cultivars in use today have some form of genetic resistance incorporated, generally against a number of diseases. This may involve single or multiple genes that are characterized as having recessive or dominant effects (Crute and Pink 1996). Without the incorporation of these resistance genes, crop productivity and yield would be substantially reduced (Agrios 1997).

With the beginning of the molecular era of plant biology in the early 1980's, a major area of research has been to identify, clone, and characterize various genes involved in disease resistance. As a result, many intriguing mechanisms, which plants have evolved to respond to pathogen infection, have been identified over the past 10 years, and remarkable progress has been made toward elucidating the multitude of genes that are involved in these responses. The identification of these genes has made it possible to subsequently evaluate their specific roles and importance in disease response pathways using transgenic plants developed with genetic engineering techniques (Fig. 1).

In this paper, I will review advances made in utilizing a broad range of cloned genes (from both plant and nonplant sources) to enhance disease resistance against fungal pathogens in transgenic plants and address future challenges and prospects. Several other reviews on the subject of genetic engineering for fungal disease resistance provide related information on this topic (Shah 1997; Swords et al. 1997; Bushnell et al. 1998; Evans and Greenland 1998; Honée 1999; Melchers and Stuiver 2000; Rommens and Kishore 2000). The approaches that have been taken by researchers can be grouped into five general categories (see Table 1):

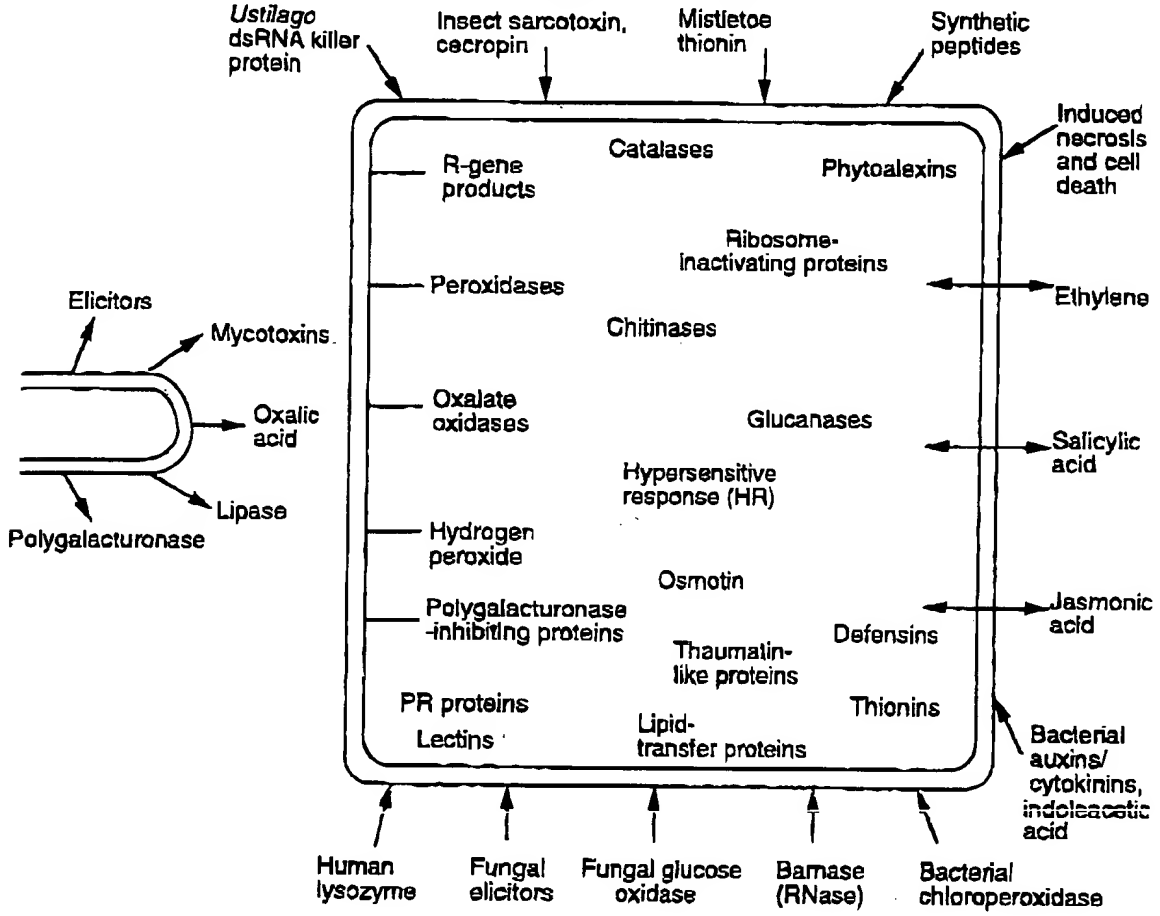
(1) The expression of gene products that are directly toxic to pathogens or that reduce their growth. These include pathogenesis-related proteins (PR proteins) such as hydrolytic enzymes (chitinases, glucanases), antifungal proteins (osmotin- and thaumatin-like), antimicrobial peptides (thionins, defensins, lectin), ribosome-inactivating proteins (RIP), and phytoalexins.
(2) The expression of gene products that destroy or neutralize a component of the pathogen arsenal such as polygalacturonase, oxalic acid, and lipase.
(3) The expression of gene products that can potentially enhance the structural defenses in the plant. These include elevated levels of peroxidase and lignin.
(4) The expression of gene products releasing signals that can regulate plant defenses. This includes the production of specific elicitors, hydrogen peroxide (H_2O_2), salicylic acid (SA), and ethylene (C_2H_4).
(5) The expression of resistance gene (R) products involved in the hypersensitive response (HR) and in interactions with avirulence (Avr) factors.

The selection of genes to genetically engineer into plants to protect against fungal diseases has been based, in part, on evaluation of the toxicity of the gene product to fungal growth or development in vitro, and to the prominence of the particular gene(s) in a disease resistance response pathway. Many gene products belong to the group of PR proteins (Neuhaus 1999; Van Loon and Van Strien 1999), while others are involved in phytoalexin biosynthetic pathways and in enhancing plant structural defenses. Although some of these gene products may normally be expressed relatively late in the response pathway, e.g. after 48 h, the rationale for developing transgenic plants was to achieve early and high expression (overexpression) of these proteins, usually constitutively throughout most of the plant. In other instances, enhanced levels of protein expression were reasoned to provide a greater inhibitory effect on fungal development than lower naturally occurring or induced levels in the plant. Other genes were selected in genetic engineering efforts for their ability to induce an array of naturally occurring defense mechanisms in the plant. More recently, the cloning of R genes has precipitated interest in utilizing these genes to provide broad-spectrum disease resistance. Some genetic engineering approaches have been based on novel approaches of introducing genes from double-stranded RNA entities from viruses found in fungi (Clausen et al. 2000) and genes of lysozymes cloned from human tissues (Nakajima et al. 1997; Takaichi and Oeda 2000) and from a range of microbes (Lorito and Scala 1999).

Hydrolytic enzymes

The most widely used approach has been to overexpress chitinases and glucanases, which belong to the group of PR proteins (Neuhaus 1999) and have been shown to exhibit antifungal activity in vitro (Boller 1993; Yun et al. 1997). Since chitins and glucans comprise major components of

Fig. 1. Transgenic plants with enhanced disease resistance have been engineered to express gene products to counterattack fungal virulence products (from hypha on left), enhanced expression of plant-derived gene products (inside of cell) or gene products from nonplant sources (outside of cell). The results from these experiments are summarized in Table 1.



the cell wall in many groups of fungi, the overexpression of these enzymes in plant cells is postulated to cause the hyphae to lyse and thereby reduce fungal growth (Mauch and Staehelin 1989). The specific roles of these hydrolases in resistance to disease have been difficult to prove in nontransgenic plants, since the enzymes are frequently encountered in both resistant and susceptible tissues, and their expression can also be induced by environmental triggers and plant senescence (Punja and Zhang 1993). However, following expression of different types of chitinases in a range of transgenic plant species, the rate of lesion development and the overall size and number of lesions were reduced upon challenge with many fungal pathogens (Table 1), including those with a broad host range, such as *Botrytis cinerea* and *Rhizoctonia solani*. However, chitinase expression was ineffective against other pathogens, such as *Cercospora nicotianae*, *Colletotrichum lagenarium*, and *Pythium* spp., indicating that differences exist in sensitivity of fungi to chitinase. The characteristics of chitinases from different sources can vary, e.g. in substrate binding specificity, pH optimum, and localization in the cell, and this can lead to differences in antifungal activity (Sela-Buurlage et al. 1993), highlighting the importance of appropriate selection of the gene to be used against a targeted pathogen or group of pathogens. While the results from these efforts have not been spectacular in terms of the level of disease control, they demonstrate that the rate of disease progress and overall disease severity can be significantly reduced. A few transgenic crop species expressing chitinases have been evaluated in field trials and it was demonstrated that disease incidence was reduced (Howie et al. 1994; Grison et al. 1996; Melchers and Stuiver 2000).

There are fewer examples of the expression of glucanases in transgenic plants (Table 1) but the results have generally been similar to that for chitinase expression. The combined expression of chitinase and glucanase in transgenic carrot, tomato, and tobacco was much more effective in preventing development of disease due to a number of pathogens than either one alone (Jongedijk et al. 1995; Van den Elzen et al. 1993; Zhu et al. 1994), confirming the synergistic activity of these two enzymes reported from in vitro studies (Sela-Buurlage et al. 1993; Van den Elzen et al. 1993; Melchers and Stuiver 2000). As a general rule, the deployment of genetic engineering approaches that involve the expression of two or more antifungal gene products in a specific crop should provide more effective and broad-spectrum disease control than the single-gene strategy (Lamb et al. 1992; Cornelissen and Melchers 1993; Strittmatter and Wegner

Table 1. Plant species genetically engineered to enhance resistance to fungal diseases (1991–2001).

Strategy used and plant species engineered	Expressed gene product	Effect on disease development	Reference
Expression of hydrolytic enzymes			
Alfalfa (*Medicago sativa* L.)	Alfalfa glucanase	Reduced symptom development due to *Phytophthora megasperma*; no effect on *Stemphylium alfalfae*	Masoud et al. (1996)
American ginseng (*Panax quinquefolius* L.)	Rice chitinase	Not tested	W.P. Chen and Z.K. Punja (unpublished data)
Apple (*Malus ×domestica* Auth.)	*Trichoderma harzianum* endochitinase	Reduced lesion number and lesion area due to *Venturia inaequalis*	Bolar et al. (2000); Wong et al. (1999)
Barley (*Hordeum vulgare* L.)	*Trichoderma* endo-1, 4-β-glucanase	Not tested	Nuutila et al. (1999)
Canola (*Brassica napus* L.)	Bean chitinase	Reduced rate and total seedling mortality due to *Rhizoctonia solani*	Broglie et al. (1991)
	Tomato chitinase	Lower percentage of diseased plants due to *Cylindrosporium concentricum* and *Sclerotinia sclerotiorum*	Grison et al. (1996)
Carrot (*Daucus carota* L.)	Tobacco chitinase	Reduced rate and final incidence of disease due to *Botrytis cinerea*, *Rhizoctonia solani*, and *Sclerotium rolfsii*: no effect on *Thielaviopsis basicola* and *Alternaria radicina*	Punja and Raharjo (1996)
Chrysanthemum (*Dendranthema grandiflorum*) (Ramat.) Kitamura	Rice chitinase	Reduced lesion development due to *Botrytis cinerea*	Takatsu et al. (1999)
Cucumber (*Cucumis sativus* L.)	Petunia and tobacco chitinases	No effect on disease development due to *Colletotrichum lagenarium* and *Rhizoctonia solani*	Punja and Raharjo (1996)
	Rice chitinase	Reduced lesion development due to *Botrytis cinerea*	Tabei et al. (1998)
Grape (*Vitis vinifera* L.)	Rice chitinase	Reduced development of *Uncinula necator* and fewer lesions due to *Elisinoe ampelina*	Yamamoto et al. (2000)
	Trichoderma harzianum endochitinase	Reduction of *Botrytis cinerea* development in preliminary tests	Kikkert et al. (2000)
Peanut (*Arachis hypogaea*)	Tobacco chitinase	Delayed lesion development and smaller lesion size due to *Cercospora arachidicola*	Rohini and Rao (2001)
Potato (*Solanum tuberosum* L.)	*Trichoderma harzianum* endochitinase	Lower lesion numbers and size due to *Alternaria solani*; reduced mortality due to *Rhizoctonia solani*	Lorito et al. (1998)
Rice (*Oryza sativa*)	Rice chitinase	Delayed onset and reduced severity of disease symptoms due to *Magnaporthe grisea*	Nishizawa et al. (1999)
	Rice chitinase	Fewer numbers of lesions and smaller size due to *Rhizoctonia solani*	Lin et al. (1995); Datta et al. (2000, 2001)
Rose (*Rosa hybrida* L.)	Rice chitinase	Reduced lesion diameter due to black spot (*Diplocarpon rosae*)	Marchant et al. (1998)
Strawberry (*Fragaria ×ananassa* Duch.)	Rice chitinase	Reduced development of powdery mildew (*Sphaerotheca humuli*)	Asao et al. (1997)
Tobacco (*Nicotiana benthamiana* L.)	Sugarbeet chitinase	No effect on *Cercospora nicotianae*	Nielsen et al. (1993)
Tobacco (*Nicotiana sylvestris* L.)	Tobacco chitinase	No effect on *Cercospora nicotianae*	Neuhaus et al. (1991)
	Tobacco chitinase	Reduced colonization by *Rhizoctonia solani*	Vierheilig et al. (1993)
Tobacco (*Nicotiana tabacum* L.)	Bean chitinase	Lower seedling mortality due to *Rhizoctonia solani*: no effect on *Pythium aphanidermatum*	Broglie et al. (1991, 1993)
Tobacco (*N. tabacum* L.)	Peanut chitinase	Not tested	Kellmann et al. (1996)

Table 1 (*continued*).

Strategy used and plant species engineered	Expressed gene product	Effect on disease development	Reference
	Serratia marcescens chitinase	Reduced disease incidence due to *Rhizoctonia solani* on seedlings; no effect on *Pythium ultimum*	Howie et al. (1994)
	Serratia marcescens chitinase	Reduced development of *Rhizoctonia solani*	Jach et al. (1992)
	Rhizopus oligosporus chitinase	Reduced rate of development and size of lesions on leaves due to *Botrytis cinerea* and *Sclerotinia sclerotiorum*	Terakawa et al. (1997)
	Streptomyces chitosanase	Not tested	El Quakfaoui et al. (1995)
	Trichoderma harzianum endochitinase	Reduced symptoms due to *Alternaria alternata*, *Botrytis cinerea*, and *Rhizoctonia solani*	Lorito et al. (1998)
	Baculovirus chitinase	Reduced lesion development due to brown spot (*Alternaria alternata*)	Shi et al. (2000)
	Soybean glucanase	Reduced development of *Phytophthora parasitica* and *Alternaria alternata*	Yoshikawa et al. (1993)
	Tobacco glucanase	Reduced disease symptoms due to *Phytophthora parasitica* and *Peronospora tabacina*	Lusso and Kuc (1996)
	Acidothermus cellulolyticus endoglucanase	Not tested	Dai et al. (2000)
Tomato (*Lycopersicon esculentum* Mill.)	Wild tomato (*Lycopersicon chilense*) chitinase	Reduced development of *Verticillium dahliae* races 1 and 2	Tabaeizadeh et al. (1999)
Wheat (*Triticum aestivum* L.)	Barley chitinase	Reduced development of colonies of *Blumeria graminis* f. sp. *tritici*	Bliffeld et al. (1999)
	Barley chitinase	Reduced development of colonies of *Blumeria graminis* and *Puccinia recondita*	Oldach et al. (2001)
Expression of pathogenesis-related (PR) proteins			
Canola (*B. napus*)	Pea chitinase, PR-10.1 gene	No effect on *Leptosphaeria maculans*	Wang et al. (1999)
	Pea defense response gene, defensin	Reduced infection and development of *Leptosphaeria maculans*	Wang et al. (1999)
Carrot (*D. carota*)	Rice TLP	Reduced rate and final disease incidence due to *Botrytis cinerea* and *Sclerotinia sclerotiorum*	W.P. Chen and Z.K. Punja (unpublished data)
Potato (*Solanum commersonii* Dun.)	Potato osmotin-like protein	Enhanced tolerance to infection by *Phytophthora infestans*	Zhu et al. (1996)
Potato (*S. tuberosum*)	Tobacco osmotin	Delayed onset and rate of disease due to *Phytophthora infestans*	Liu et al. (1994)
	Pea PR-10 gene	Reduced development of *Verticillium dahliae*	Chang et al. (1993)
	Potato defense response gene *STH-2*	No effect against *Phytophthora infestans*	Constabel et al. (1993)
Rice (*O. sativa*)	Rice *Rir1b* defense gene	Fewer lesions due to *Magnaporthe grisea*	Schaffrath et al. (2000)
	Rice TLP	Reduced lesion development due to *Rhizoctonia solani*	Datta et al. (1999)
Tobacco (*N. tabacum*)	Tobacco PR-1a	Reduced rate and final disease due to *Peronospora tabacina* and *Phytophthora parasitica*	Alexander et al. (1993)
	Tobacco osmotin	No effect on *Phytophthora parasitica* var. *nicotianae*	Liu et al. (1994)

Table 1 (*continued*).

Strategy used and plant species engineered	Expressed gene product	Effect on disease development	Reference
Wheat (*T. aestivum*)	*Aspergillus giganteus* antifungal protein	Reduced development of colonies of *Blumeria graminis* and *Puccinia recondita*	Oldach et al. (2001)
	Rice TLP	Delayed development of *Fusarium graminearum*	Chen et al. (1999)
Expression of antimicrobial proteins, peptides, or compounds			
Arabidopsis thaliana L.	*Arabidopsis* thionin	Reduced development and colonization by *Fusarium oxysporum*	Epple et al. (1997)
	Mistletoe thionin viscotoxin	Reduced infection and development of *Plasmodiophora brassicae*	Holtorf et al. (1998)
Carrot (*D. carota*)	Human lysozyme	Enhanced resistance to *Erysiphe heraclei* and *Alternaria dauci*	Takaichi and Oeda (2000)
Geranium (*Pelargonium* sp.)	Onion antimicrobial protein	Reduced development and sporulation of *Botrytis cinerea*	Bi et al. (1999)
Potato (*S. tuberosum*)	Alfalfa defensin	Enhanced resistance to *Verticillium dahliae*	Gao et al. (2000)
	Bacillus amyloliquefaciens barnase (RNase)	Delayed sporulation and reduced sporangia production by *Phytophthora infestans*	Strittmatter et al. (1995)
	Synthetic cationic peptide chimera	Reduced development of *Fusarium solani* and *Phytophthora cactorum*	Osusky et al. (2000)
	Human lactoferrin	Not tested	Chong and Langridge (2000)
Rice (*O. sativa*)	Maize RIP	No effect on *Magnaporthe grisea* or *Rhizoctonia solani*	Kim et al. (1999)
Tobacco (*N. tabacum*)	*Amaranthus* hevein-type peptide, *Mirabilis* knottin-type peptide	No effect on *Alternaria longipes* or *Botrytis cinerea*	De Bolle et al. (1996)
	Radish defensin	Reduced infection and lesion size due to *Alternaria longipes*	Terras et al. (1995)
	Barley RIP	Reduced incidence and severity of *Rhizoctonia solani*	Logemann et al. (1992)
	Maize RIP	Lower damage due to *Rhizoctonia solani*	Maddaloni et al. (1997)
	Pokeweed antiviral protein	Lower rate of infection and mortality due to *Rhizoctonia solani*	Wang et al. (1998); Zoubenko et al. (1997)
	Sarcotoxin peptide from *Sarcophaga peregrina*	Enhanced seedling survival following inoculation with *Rhizoctonia solani*, *Pythium aphanidermatum*, and *Phytophthora nicotianae*	Mitsuhara et al. (2000)
	Stinging nettle (*Urtica dioica* L.) isolectin	Not tested	Does et al. (1999)
	Antifungal (killing) protein from virus infecting *Ustilago maydis* (dsRNA)	Not tested	Park et al. (1996)
	Chloroperoxidase from *Pseudomonas pyrrocinia*	Reduced lesion development by *Colletotrichum destructivum*	Rajasekaran et al. (2000)
	Synthetic antimicrobial peptide	Reduced lesion size due to *Colletotrichum destructivum*	Cary et al. (2000)
	Synthetic magainin-type peptide	Reduced lesion size and sporulation due to *Peronospora tabacina*	Li et al. (2001)
	Human lysozyme	Reduced colony size and conidial production by *Erysiphe cichoracearum*	Nakajima et al. (1997)
Tomato (*L. esculentum*)	Radish defensin	Reduced number and size of lesions due to *Alternaria solani*	Parashina et al. (2000)

Table 1 (*continued*).

Strategy used and plant species engineered	Expressed gene product	Effect on disease development	Reference
Wheat (*T. aestivum*)	Barley RIP	Slightly reduced development of *Blumeria graminis*	Bieri et al. (2000)
	Antifungal (killing) protein from from virus infecting *Ustilago maydis* (dsRNA)	Inhibition of *Ustilago maydis* and *Tilletia tritici* development on seeds	Clausen et al. (2000)
Expression of phytoalexins			
Alfalfa (*M. sativa*)	Alfalfa isoflavone O-methyltransferase	Reduced lesion size due to *Phoma medicaginis*	He and Dixon (2000)
	Peanut resveratrol synthase	Reduced lesion size and sporulation of *Phoma medicaginis*	Hipskind and Paiva (2000)
Barley (*H. vulgare*)	Grape stilbene (resveratrol) synthase	Reduced colonization by *Botrytis cinerea*	Leckband and Lörz (1998)
Rice (*O. sativa*)	Grape stilbene (resveratrol) synthase	Reduced lesion development due to *Pyricularia oryzae*	Stark-Lorenzen et al. (1997)
Tobacco (*N. tabacum*)	*Fusarium* trichodiene synthase	Not tested	Zook et al. (1996)
	Grape stilbene (resveratrol) synthase	Reduced colonization by *Botrytis cinerea*	Hain et al. (1993)
Tomato (*L. esculentum*)	Grape stilbene (resveratrol) synthase	Reduced lesion development by *Phytophthora infestans*; no effect on *Alternaria solani* or *Botrytis cinerea*	Thomzik et al. (1997)
Wheat (*T. aestivum*)	Grape stilbene (resveratrol) synthase	Not tested	Fettig and Hess (1999)
Inhibition of pathogen virulence products			
Canola (*B. napus*)	Barley oxalate oxidase	Not tested	Thompson et al. (1995)
Poplar (*Populus* ×*euramericana* Auth.)	Wheat oxalate oxidase	Delayed development of *Septoria musiva*	Liang et al. (2001)
Tobacco (*N. tabacum*)	*Fusarium* trichothecene-degrading enzyme	Not tested	Muhitch et al. (2000)
	Wheat oxalate oxidase (germin)	Not tested	Berna and Bernier (1997)
Tomato (*L. esculentum*)	Bean polygalacturonase inhibiting protein	No effect on disease due to *Fusarium oxysporum*, *Botrytis cinerea*, and *Alternaria solani*	Desiderio et al. (1997)
	Pear polygalacturonase inhibiting protein	Reduced rate of development of *Botrytis cinerea*	Powell et al. (2000)
	Collybia velutipes oxalate decarboxylase	Enhanced resistance to *Sclerotinia sclerotiorum*	Kesarwani et al. (2000)
Alteration of structural components			
Potato (*S. tuberosum*)	Cucumber peroxidase	No effect on disease due to *Fusarium sambucinum* and *Phytophthora infestans*	Ray et al. (1998)
Tomato (*L. esculentum*)	Tobacco anionic peroxidase	No effect on disease due to *Fusarium oxysporum* and *Verticillium dahliae*	Lagrimini et al. (1993)
Wheat (*T. aestivum*)	Wheat germin (no oxalate oxidase activity)	Reduced penetration by *Blumeria graminis* into epidermal cells	Schweizer et al. (1999)
Regulation of plant defense responses			
A. thaliana	*Arabidopsis* NPR1 protein	Reduced infection and growth of *Peronospora parasitica*	Cao et al. (1998)

Table 1 (*concluded*).

Strategy used and plant species engineered	Expressed gene product	Effect on disease development	Reference
Cotton (*Gossypium hirsutum* L.), tobacco (*N. tabacum*)	*Talaromyces flavus* glucose oxidase	Enhanced protection against *Rhizoctonia solani* and *Verticillium dahliae*; no effect on *Fusarium oxysporum*	Murray et al. (1999)
Potato (*S. tuberosum*)	*Aspergillus niger* glucose oxidase	Delayed lesion development due to *Phytophthora infestans*; reduced disease development due to *Alternaria solani* and *Verticillium dahliae*	Wu et al. (1995, 1997)
	Tobacco catalase	Reduced lesion size due to *Phytophthora infestans*	Yu et al. (1999)
	Bacterial salicylate hydroxylase	No effect on *Phytophthora infestans*	Yu et al. (1997)
Tobacco (*N. tabacum*), *A. thaliana*	Bacterial salicylate hydroxylase	Enhanced susceptibility to *Phytophthora parasitica*, *Cercospora nicotianae*, and *Peronospora parasitica*	Delaney et al. (1994); Donofrio and Delaney (2001)
Tobacco (*N. tabacum*)	Bacterial enzymes generating salicylic acid	Enhanced resistance to *Oidium lycopersicon*	Verberne et al. (2000)
	Arabidopsis ethylene-insensitivity gene	Enhanced susceptibility to *Pythium sylvaticum*	Knoester et al. (1998)
	Phytophthora cryptogea elicitor (β-cryptogein)	Reduced infection by *Phytophthora parasitica*	Tepfer et al. (1998)
	Phytophthora cryptogea elicitor (cryptogein)	Enhanced resistance to *Phytophthora parasitica*, *Thielaviopsis basicola*, *Botrytis cinerea*, and *Erysiphe cichoracearum*	Keller et al. (1999)
Expression of combined gene products			
Carrot (*D. carota*)	Tobacco chitinase + β-1,3-glucanase, osmotin	Enhanced resistance to *Alternaria dauci*, *Alternaria radicina*, *Cercospora carotae*, and *Erysiphe heraclei*	Melchers and Stuiver (2000)
Tobacco (*N. tabacum*)	Barley chitinase + β-1,3-glucanase, or chitinase + RIP	Reduced disease severity due to *Rhizoctonia solani*	Jach et al. (1995)
	Rice chitinase + alfalfa glucanase	Reduced rate of lesion development and fewer lesions due to *Cercospora nicotianae*	Zhu et al. (1994)
Tomato (*L. esculentum*)	Tobacco chitinase + β-1,3-glucanase	Reduced disease severity due to *Fusarium oxysporum* f. sp. *lycopersici*	Jongedijk et al. (1995); Van den Elzen et al. (1993)

Note: dsRNA, double-stranded RNA; RIP, ribosome-inactivating protein; TLP, thaumatin-like protein.

1993; Jach et al. 1995; Shah 1997; Evans and Greenland 1998; Salmeron and Vernooij 1998; Melchers and Stuiver 2000).

Pathogenesis-related proteins

Other PR proteins that exhibit antifungal activity, including osmotin- and thaumatin-like proteins (TLP), and some uncharacterized PR proteins have been engineered into crop plants (Table 1). Osmotin is a basic 24-kDa protein belonging to the PR-5 family whose members have a high degree of homology to the sweet-tasting protein thaumatin from *Thaumatococcus danielli* and are produced in plants under different stress conditions (Zhu et al. 1995). The PR-5 proteins induce fungal cell leakiness, presumably through a specific interaction with the plasma membrane that results in the formation of transmembrane pores (Kitajima and Sato 1999). Osmotin has been shown to have antifungal activity in vitro (Woloshuk et al. 1991; Melchers et al. 1993; Liu et al. 1994) and, when tested in combination with chitinase and β-1,3-glucanase, showed enhanced lytic activity (Lorito et al. 1996). When expressed in transgenic potato, osmotin was shown to delay expression of disease symptoms caused by *Phytophthora infestans* (Table 1). Thaumatin-like proteins are also expressed in plants in response to a range of stress conditions and were demonstrated to have antifungal activity in vitro (Malehorn et al. 1994; Koiwa et al. 1997). Expression of TLP in transgenic plants was reported to delay disease development due to several pathogens, including *Botrytis*, *Fusarium*, *Rhizoctonia*, and *Sclerotinia* (Table 1). Combinations of PR-5 protein expression with chitinases or glucanases in transgenic plants have not been reported and it is anticipated that the level of disease reduction achieved would be significantly higher.

Antimicrobial proteins, peptides, and other compounds

Defensins and thionins are low molecular mass (around 5 kDa) cysteine-rich peptides (45–54 amino acids in length)

found in monocotyledonous and dicotyledonous plant species, which were initially derived from seeds and have antimicrobial activity (Bohlmann 1994; Broekaert et al. 1995; Evans and Greenland 1998). Viscotoxin from mistletoe (*Viscum album*) is a thionin. It was proposed that these peptides play a role in protecting seeds from infection by pathogens (Broekaert et al. 1997). Defensins are also found in insects and mammals, where they play an important role in curtailing or limiting microbial attack (Rao 1995). These peptides may exert antifungal activity by altering fungal membrane permeability and (or) inhibiting macromolecule biosynthesis, and thionins may be toxic to plant and animal cell cultures as well (Broekaert et al. 1997). The overexpression of defensins and thionins in transgenic plants was demonstrated to reduce development of several different pathogens, including *Alternaria*, *Fusarium*, and *Plasmodiophora* (Table 1), and provided resistance to *Verticillium* on potato under field conditions (Gao et al. 2000).

Chitin-binding peptides (hevein- and knottin-types) are 36–40 residues in length and have been recovered from the seeds of some plant species. They contain cysteine residues and were demonstrated to have antifungal activity in vitro (Broekaert et al. 1997). However, expression of *Amaranthus* hevein-type peptide and *Mirabilis* knottin-type peptide in transgenic tobacco did not enhance tolerance to *Alternaria longipes* or *Botrytis cinerea* (De Bolle et al. 1996). It was postulated that the presence of cations, particularly Ca^{2+}, may have inhibited the activity of these peptides in vivo. Modifications to amino acid sequences of peptides may enhance the antifungal activity (Evans and Greenland 1998).

Ribosome-inactivating proteins are plant enzymes that have 28 S rRNA *N*-glycosidase activity, which depending on their specificity, can inactivate conspecific or foreign ribosomes, thereby shutting down protein synthesis. The most common cytosolic type I RIP from the endosperm of cereal grains do not act on plant ribosomes but can affect foreign ribosomes, such as those of fungi (Stirpe et al. 1992; Hartley et al. 1996). Expression of barley seed RIP reduced development of *Rhizoctonia solani* in transgenic tobacco (Logemann et al. 1992) but had little effect on *Blumeria graminis* in transgenic wheat (Bieri et al. 2000). In the latter study, the RIP was targeted to the apoplastic space and may have had less activity against development of the intracellular haustoria of the mildew pathogen. It has been demonstrated that combined expression of chitinase and RIP in transgenic tobacco had a more inhibitory effect on *Rhizoctonia solani* development than the individual proteins (Jach et al. 1995). Therefore, dissolution of the fungal cell wall by hydrolytic enzymes should enhance the efficacy of antifungal proteins and peptides in transgenic plants. Human lysozyme has lytic activity against fungi and bacteria, and when expressed in transgenic carrot and tobacco, enhanced resistance to several pathogens, including *Erysiphe* and *Alternaria* (Nakajima et al. 1997; Takaichi and Oeda 2000). An antimicrobial protein with homology to lipid transfer protein was shown to reduce development of *Botrytis cinerea* when expressed in transgenic geranium (Bi et al. 1999).

Pokeweed (*Pytolacca americana*) antiviral protein with type I RIP activity has been expressed in transgenic tobacco and shown to reduce development of *Rhizoctonia solani* (Wang et al. 1998). Because of some toxicity to plant cells, nontoxic mutant proteins were derived and their expression in transgenic plants led to the activation of defense-related signalling pathways and PR-protein induction (e.g., chitinase and glucanase), which in turn enhanced plant resistance to infection by *Rhizoctonia solani* (Zoubenko et al. 1997). The induction of defense pathways in transgenic plants using other strategies will be discussed later.

Antimicrobial peptides have been synthesized in the laboratory to produce smaller (10–20 amino acids in length) molecules that have enhanced potency against fungi (Cary et al. 2000). In addition, a synthetic cationic peptide chimera (cecropin–melittin) with broad-spectrum antimicrobial activity has been produced (Osusky et al. 2000). When expressed in transgenic potato and tobacco, these synthetic peptides have provided enhanced resistance against a number of fungal pathogens, including *Colletotrichum*, *Fusarium*, and *Phytophthora* (Table 1). These peptides may demonstrate lytic activity against fungal hyphae, inhibit cell wall formation, and (or) enhance membrane leakage. The ability to create synthetic recombinant and combinatorial variants of peptides that can be rapidly screened in the laboratory could provide additional opportunities to engineer resistance to a range of pathogens simultaneously. Enhancement of the specific activities of antifungal enzymes or the creation of variants with broad activity using directed molecular evolution (DNA shuffling) has also been proposed as a method to enhance the efficacy of transgenic plants in the future (Lassner and Bedbrook 2001).

Phytoalexins

These are low molecular mass secondary metabolites produced in a broad range of plant species, which were demonstrated to have antimicrobial activity and are induced by pathogen infection and elicitors (Hammerschmidt 1999; Grayer and Kokubun 2001). Phytoalexins are synthesized through complex biochemical pathways (Dixon et al. 1996), such as the shikimic acid pathway, and genetic manipulation of these pathways to suppress or enhance phytoalexin production has been difficult to achieve. Similar to the hydrolytic enzymes, it has not been easy to conclusively demonstrate the role played by phytoalexins in enhancing resistance to disease in many host–pathogen interactions. A mutant of *Arabidopsis* deficient in the production of the indole-type phytoalexin camalexin was shown to be more susceptible to infection by *Alternaria brassicicola* but not to *Botrytis cinerea* (Thomma et al. 1999b). Using transgenic plants, it has been possible to also show that the overexpression of genes encoding certain phytoalexins, such as *trans*-resveratrol and medicarpin, resulted in delayed development of disease and symptom production by a number of pathogens on several plant species (Table 1). These studies are encouraging in light of the difficulties of engineering the complex biochemical pathways leading to phytoalexin accumulation in plants (Dixon et al. 1996).

Inhibition of pathogen virulence products

The plant cell wall acts as a barrier to penetration by fungal pathogens and numerous strategies have evolved among plant pathogens to overcome this (Walton 1994). These include se-

cretion of a range of plant cell wall degrading enzymes (depolymerases) and the production of toxins such as oxalic acid by fungal pathogens. Several strategies to engineer resistance against fungal infection have targeted the inactivation of these pathogen virulence products. Polygalacturonase-inhibiting proteins (PGIP) are glycoproteins present in the cell wall of many plants and that can inhibit the activity of fungal endopolygalacturonases (Powell et al. 1994; Desiderio et al. 1997). The expression of PGIP in transgenic plants led to contrasting results: in transgenic tomato expressing a bean PGIP, resistance to *Fusarium*, *Botrytis*, or *Alternaria* was not enhanced (Desiderio et al. 1997) while in transgenic tomato expressing a pear PGIP, colonization of leaves and fruits by *Botrytis* was reduced (Powell et al. 2000). In the former study, it was shown that PGIPs from bean differed in specificity to fungal polygalacturonase in vitro, and the PGIP-1 that had been selected for transformation was not inhibitory (Desiderio et al. 1997). Thus, appropriate in vitro screening of PGIPs would be required prior to undertaking transformation experiments. As with the PR proteins and antifungal compounds, disease development was reduced by PGIPs but not totally prevented in the transgenic plants.

Another developed strategy that could have potential to reduce pathogen infection is immunomodulation, the expression of genes encoding antibodies or antibody fragments in plants (plantibodies) that could bind to pathogen virulence products (De Jaeger et al. 2000; Schillberg et al. 2001). The antibodies can be expressed inter- or extracellularly and can bind to and inactivate enzymes, toxins, or other pathogen factors involved in disease development. Currently, there are no published reports on the expression of antifungal antibodies in transgenic plants that have led to a reduction in disease. However, it has been demonstrated that antilipase antibodies inhibited infection of tomato by *Botrytis cinerea*, when mixed with spore inoculum, by preventing fungal penetration through the cuticle (Comménil et al. 1998). Similarly, infection by *Colletotrichum gloeosporioides* on various fruits was inhibited using polyclonal antibodies that bound to fungal pectate lyase (Wattad et al. 1997). Genetic engineering of antibody expression in plants is extremely challenging technically and the applications to fungal disease control (immunization) have yet to be determined, although success against virus diseases has been reported (De Jaeger et al. 2000).

Production of phytotoxic metabolites, such as mycotoxins and oxalic acid, by fungal pathogens has been shown to facilitate infection of host tissues following cell death. Degradation of these compounds by enzymes expressed in transgenic plants could provide an opportunity to enhance resistance to disease. Expression of a trichothecene-degrading enzyme from *Fusarium sporotrichioides* in transgenic tobacco reduced plant tissue damage and enhanced seedling emergence in the presence of the trichothecene (Muhitch et al. 2000). The effect on pathogen development was not tested. Germin-like oxalate oxidases are stable glycoproteins first discovered in cereals, which are present during seed germination and are induced in response to fungal infection and abiotic stress (Dumas et al. 1995; Zhang et al. 1995; Berna and Bernier 1997). Their activity on the substrate oxalic acid results in the production of CO_2 and H_2O_2; the latter can induce defense responses in the plant and enhance strengthening of cell walls (Brisson et al. 1994; Mehdy 1994). The expression of barley oxalate oxidase in oilseed rape enhanced tolerance to the phytotoxic effects of oxalic acid, although the effect on the target pathogen *Sclerotinia sclerotiorum* was not evaluated (Thompson et al. 1995). Expression of oxalate oxidase in transgenic hybrid poplar enhanced resistance to *Septoria*, while oxalate decarboxylase expression enhanced resistance of tomato to *Sclerotinia sclerotiorum* (Table 1). These reports indicate that the inactivation of specific pathogen virulence factors, such as toxins, by gene products expressed in transgenic plants has the potential to reduce development of specific fungal pathogens.

Alteration of structural components

Lignification of plant cells around sites of infection or lesions has been reported to be a defense response of plants that can potentially slow down pathogen spread (Nicholson and Hammerschmidt 1992). The enzyme peroxidase is required for the final polymerization of phenolic derivatives into lignin and may also be involved in suberization or wound healing. A decrease in polyphenolic compounds, such as lignin, in potato tubers by redirection of tryptophan in transgenic plants through expression of tryptophan decarboxylase rendered tissues more susceptible to *Phytophthora infestans* (Yao et al. 1995), illustrating the role of phenolic compounds in defense. Reduction of phenylpropanoid metabolism through inhibition of phenylalanine ammonia-lyase activity in transgenic tobacco also rendered tissues more susceptible to *Cercospora nicotianae* (Maher et al. 1994). Overexpression of a cucumber peroxidase gene in transgenic potato, however, did not increase resistance of tissues to infection by *Fusarium* or *Phytophthora*, and lignin levels were not significantly affected, in spite of elevated peroxidase expression (Ray et al. 1998). It was suggested that peroxidase levels may not have been the limiting step for lignification or that the native peroxidase activity may have been cosuppressed. Overexpression of a tobacco anionic peroxidase gene in tomato did enhance lignin levels but resistance to fungal pathogens was not enhanced (Lagrimini et al. 1993). Lignin levels were also significantly higher following expression of the H_2O_2-generating enzyme glucose oxidase in transgenic potato (Wu et al. 1997) and by expression of the hormone indoleacetic acid (IAA) in transgenic tobacco (Sitbon et al. 1999). In the former case, tolerance to several fungal pathogens was enhanced (Table 1). Peroxidase overexpression in plants can, however, have negative effects on plant growth and development (Lagrimini et al. 1997), and the results to date indicate that this approach appears to hold less promise for enhancing disease resistance.

A reduction in large callose deposits surrounding haustoria of *Peronospora parasitica* infecting *Arabidopsis thaliana* was indirectly achieved in transgenic plants not accumulating SA by expression of the enzyme salicylate hydroxylase (Donofrio and Delaney 2001). These plants also had reduced expression of the *PR-1* gene and exhibited significantly enhanced susceptibility to the pathogen, suggesting that callose deposition during normal defense responses of the plant was influenced by the reduced levels of SA.

Activation of plant defense responses

One activator of host defense responses are elicitor molecules from an invading pathogen. These can trigger a network of signalling pathways that coordinate the defense responses of the plant, including HR, PR protein, and phytoalexin production (Heath 2000; McDowell and Dangl 2000; Shirasu and Schulze-Lefert 2000). A gene encoding the elicitor cryptogein (a small basic protein, 98 amino acids in length) from the pathogen *Phytophthora cryptogea* was cloned and expressed in transgenic tobacco under control of a pathogen-inducible promoter (Keller et al. 1999). Challenge inoculation with a range of fungi induced the HR as well as several defense genes, and growth of the pathogens was concomitantly restricted (Table 1). Resistance to the pathogens was not complete, possibly because of the time needed for production of the transgenic elicitor following initial infection (Keller et al. 1999). Another elicitor, INF1, was shown to act as an Avr factor in the tobacco – *Phytophthora infestans* interaction and triggered the onset of the HR (Kamoun et al. 1998). Expression of the gene encoding the AVR9 peptide elicitor from *Cladosporium fulvum* in transgenic tomatoes containing the *Cf-9* gene resulted in a necrotic defense response (Hammond-Kosack et al. 1994; Honée et al. 1995). The development of lesions resembling the HR induced through expression of a bacterial proton pump gene (bacterio-opsin) from *Halobacterium halobium* activated multiple defense systems in transgenic tobacco plants (Mittler et al. 1995) in the absence of pathogen challenge. In transgenic potato, expression of bacterio-opsin enhanced resistance to some pathogens but had no effect on others (Abad et al. 1997), while in poplar, there was no effect on disease development (Mohamed et al. 2001). Antisense inhibition of catalase, a H_2O_2-degrading enzyme, resulted in development of necrotic lesions and PR-protein accumulation (Takahashi et al. 1997). While these and other reports indicate that induction of the HR and necrosis, with the resulting activation of general defense pathways, could potentially result in broad-spectrum disease resistance (Bent 1996; Honée 1999; Melchers and Stuiver 2000), the use of such an approach would require tight regulation of the expressed phenotype, in addition to ensuring that no deleterious side effects, such as abnormal or suppressed growth, occurred on the transgenic plants. If successful, the activation of general defense responses in these transgenic plants would provide protection against viral and bacterial pathogens in addition to fungi.

Another activator of defense responses that has been engineered in transgenic plants is H_2O_2 generated through expression of genes encoding for glucose oxidase (Table 1). H_2O_2 has been shown to directly inhibit pathogen growth (Wu et al. 1995) and to induce PR proteins, SA, and ethylene (Wu et al. 1997; Chamnongpol et al. 1998), as well as phytoalexins (Mehdy 1994). It is produced during the early oxidative burst in plant cell response to infection (Baker and Orlandi 1995) and can trigger the HR (Levine et al. 1994; Tenhaken et al. 1995), strengthen cell walls (Brisson et al. 1994), and enhance lignin formation (Wu et al. 1997). Expression of elevated levels of H_2O_2 in transgenic cotton, tobacco, and potato reduced disease development due to a number of different fungi, including *Rhizoctonia*, *Verticillium*, *Phytophthora*, and *Alternaria* (Table 1); high levels can, however, be phytotoxic (Murray et al. 1999). In one study, necrotic lesions from the HR enhanced infection by the necrotrophic pathogen *Botrytis cinerea* (Govrin and Levine 2000). Therefore, the widespread induction of cell death in a transgenic plant to induce disease resistance has to be approached with caution.

Other activators of plant defense responses include signalling molecules such as SA, ethylene, and jasmonic acid (Yang et al. 1997; Dong 1998; Reymond and Farmer 1998; Dempsey et al. 1999). The roles of SA as a signal molecule for the activation of plant defense responses to pathogen infection and as an inducer of systemic acquired resistance (SAR) have been extensively studied (Ryals et al. 1996; Stichter et al. 1997; Dempsey et al. 1999; Métraux 2001). Using transgenic plants, evidence for the role of SA in defense response activation has been obtained. Plants expressing the SA-metabolizing enzyme salicylate hydroxylase, a bacterial protein that converts SA to the inactive form catechol, did not accumulate high levels of SA and had enhanced susceptibility to pathogen infection (Gaffney et al. 1993; Delaney et al. 1994; Donofrio and Delaney 2001) or had unaltered susceptibility (Yu et al. 1997). A mutant of *Arabidopsis* nonresponsive to induction of SAR showed enhanced susceptibility to fungal infection (Delaney et al. 1995; Donofrio and Delaney 2001). The overexpression of SA in transgenic tobacco was recently shown to enhance PR-protein production and provide resistance to fungal pathogens (Verberne et al. 2000). Expression of tobacco catalase, an enzyme with SA-binding activity, in transgenic potato enhanced defense gene expression leading to SAR and enhanced tolerance to *Phytophthora infestans* (Yu et al. 1999). Overexpression of the *NPR1* gene, which regulates the SA-mediated signal leading to SAR induction, in transgenic *Arabidopsis* increased the level of PR proteins during infection and enhanced resistance to *Peronospora parasitica* (Cao et al. 1998). It was postulated that synergistic interactions between PR proteins and products of other downstream defense-related genes provided the enhanced resistance. In addition, *NPR1* was only activated upon infection or by induction of SAR, avoiding potential side effects on plant growth from constitutive expression. These studies demonstrate that manipulation of SA levels in transgenic plants has the potential to lead to enhanced disease resistance by inducing PR-protein expression and other defense gene products.

Ethylene and jasmonic acid appear to be signals used in response of plants to necrotrophic pathogen attack (in contrast to biotrophic infection) and that work independently of, and possibly antagonistic to, SA-mediated responses (Dong 1998; Thomma et al. 1999a; McDowell and Dangl 2000; Lee et al. 2001). Mutant or transformed plants nonresponsive to either jasmonate or ethylene were found to be more susceptible to infection by root- and foliar-infecting fungi (Knoester et al. 1998; Staswick et al. 1998; Vijayan et al. 1998; Hoffmann et al. 1999; Thomma et al. 1999a), confirming a role for these signals in certain host–pathogen interactions. In contrast, ethylene-insensitive mutants may exhibit reduced disease symptoms, as described for *Fusarium oxysporum* on tomato (Lund et al. 1998). Genetic engineering efforts to alter ethylene or jasmonate pro-

duction in plants may, however, result in unpredictable effects on disease response, depending on the pathogen, as well as induce potential side effects in view of the multiple roles played by these signal molecules in plants (O'Donnell et al. 1996; Weiler 1997; Wilkinson et al. 1997).

Ethylene production and extracellular PR-protein expression were found to be induced by expression of cytokinins in transgenic tomato cells (Bettini et al. 1998). The engineering of hormone biosynthetic gene expression in transgenic plants has been accomplished (Hedden and Phillips 2000). Whether or not reduced or elevated levels of hormones, such as auxins, cytokinins, gibberellins, and jasmonate, can lead to the development of transgenic plants with enhanced disease resistance remains to be seen, given their broad range of physiological effects on plant development. Interestingly, inhibition of IAA production by antisense transformation of the nitrilase 1 gene in *Arabidopsis* reduced levels of IAA and development of root galls due to *Plasmodiophora brassicae* (Neuhaus et al. 2000). In contrast, overexpression of IAA in tobacco enhanced ethylene production and peroxidase activity and increased lignin content although the response to disease was not tested (Sitbon et al. 1999). Altered auxin or cytokinin expression has the potential to also affect mycorrhizal colonization of plant roots (Barker and Tagu 2000).

Resistance genes

Resistance gene products may serve as receptors for pathogen Avr factors or recognize the Avr factor indirectly through a coreceptor (Staskawicz et al. 1995). This gene-for-gene interaction triggers one or more signal transduction pathways that in turn activate defense responses in the plant to prevent pathogen growth (Hammond-Kosack and Jones 1996; De Wit 1997). These defense responses include the development of the HR, expression of PR proteins, and accumulation of SA and can lead to the development of SAR (Ryals et al. 1996; Dempsey et al. 1999; Kombrink and Schmelzer 2001). Ethylene and jasmonic acid may also be involved in signalling the defense responses in the gene-for-gene interaction (Deikman 1997; Dong 1998). Efforts to clone an array of R genes involved in fungal disease resistance have met with some success (Bent 1996; Crute and Pink 1996; Baker et al. 1997; De Wit 1997; Hammond-Kosack and Jones 1997; Ellis and Jones 1998;). The R-gene products that have been cloned from tomato, tobacco, rice, flax, *Arabidopsis*, and several other plant species shared one or more similar motifs: a serine or threonine kinase domain, a nucleotide binding site, a leucine zipper, or a leucine-rich repeat region, all of which may contribute to recognition specificity (Shirasu and Schulze-Lefert 2000; Takken and Joosten 2000). The *Hm1* R gene cloned from maize is an exception, as it encodes for a NADPH-dependent reductase that inactivates the potent toxin produced by race 1 strains of *Cochliobolus carbonum* (Johal and Briggs 1992). Many R genes belong to tightly linked multigene families, e.g. *Cf-4* to *Cf-9* encoding resistance to *Cladosporium fulvum* mold of tomato (Thomas et al. 1997).

There are several examples of the expression of R genes in transgenic plants. The overexpression of the *HRT* gene, which controls the HR to turnip crinkle virus in *Arabidopsis*, did not confer enhanced resistance to *Peronospora tabacina* (Cooley et al. 2000). The authors proposed that multiple factors may be involved in determining the resistance response, or that the resistance may be HR-independent. Expression of the *Cf-9* gene, which confers resistance in tomato to races of *Cladosporium fulvum*, in transgenic tobacco and potato gave rise to the HR when challenged with AVR9 peptide (Hammond-Kosack et al. 1998), indicating that the Cf-9 gene product was produced. However, for the disease resistance mediated by R gene – Avr factor to be fully expressed, several additional loci may be required (Hammond-Kosack and Jones 1996; Baker et al. 1997), in addition to elevated levels of SA (Delaney et al. 1994). Results to date suggest that the expression of cloned R genes in heterologous transgenic plants is unlikely by itself to enhance tolerance to fungal pathogens because of the complexity of the interacting signalling pathways. A combination of several interacting genes, similar to that for the antifungal proteins, will likely be required. An enhanced understanding of R-gene structure and function could, however, make it possible to modify functional domains in the future to tailor R genes for use in providing broad-spectrum resistance to diseases in transgenic plants (Bent 1996; Dempsey et al. 1998). Other potential approaches to the use of R genes for engineering disease resistance in plants are discussed by Rommens and Kishore (2000).

Scientific challenges

Besides identifying and cloning potentially useful genes to engineer into plants, the development of transgenic plants with enhanced fungal disease resistance faces additional challenges. Depending on the plant species, transformation frequencies can be as low as 1–10%, and out of hundreds of confirmed transgenic lines, only a few may have appropriate transgene expression levels. Recent advances in plant transformation should provide new opportunities to overcome some of these difficulties (Gelvin 1998; Hansen and Wright 1999; Newell 2000). The positive relationship of high levels of PR proteins and antifungal compounds with enhanced disease resistance in plants has been documented in many but not all cases. However, as indicated previously, there are a number of examples where transgene products expressed at high levels induced plant cell damage or had other undesirable effects. These include the engineered expression of thionins, RIP, peroxidase, H_2O_2, elicitor molecules, and growth regulators. In most instances, constitutive promoters have been used to achieve high expression levels throughout most tissues of the plant. In crops affected by pathogens that colonize more than one type of organ, e.g. roots and leaves, this is advantageous. In instances where only specific tissues need to be protected, e.g. leaves, fruit, or seed, or where the antifungal compounds need to be expressed at certain targeted sites in the cell, specific promoters would need to be identified (Bushnell et al. 1998; Dahleen et al. 2001). Wound-inducible and pathogen-inducible promoters, which have advantages for engineering specific disease resistance against fungal pathogens by expressing antifungal compounds only at sites of infection or wounds, have also been described (Roby et al. 1990; Strittmatter et al. 1995; Keller et al. 1999). Targeting of the

engineered protein to the apoplastic space or to the vacuole has been achieved in numerous previous studies and may enhance the antifungal activity, depending on the mode of infection of the pathogen. Future research will require the fine tuning of engineered gene expression and establishment of the optimal expression levels and target site in the cell needed to prevent pathogen infection.

Commercial development

Compared to the demonstrable scientific and economic success in engineering crop plants for resistance to herbicides, insect pests, and virus diseases (Shah et al. 1995), engineering of plants with enhanced disease resistance has lagged behind. Despite close to 100 published scientific reports (Table 1), of which about 30% are on tobacco used as a model system, only a few of the transgenic crops have been field-tested, and wide-scale deployment may not yet be realized for another 5–10 years. Development of transgenic plants with enhanced disease resistance is also being actively pursued in the private sector, and recent unpublished developments may not be included here. It remains to be demonstrated under field conditions to which level disease resistance is achieved and whether it is against a range of phytopathogens or only specific diseases. It is noteworthy that many of the successfully controlled pathogens in laboratory and greenhouse evaluations are those with a wide host range, such as *Rhizoctonia solani* and *Botrytis cinerea*, for which there are few available sources of resistance through conventional breeding in most crops. This is particularly true also for seedling-infecting pathogens, for which there are few examples of genetic resistance in the host. Therefore, genetic engineering of novel disease resistance traits in crop plants has the potential to provide control of devastating pathogens with reduced fungicide applications. Expression of an antifungal trait throughout the growing season, from seed to harvest, under prolonged disease-conducive conditions, can also provide significant advantages for disease management using this technology.

Issues to be addressed

The current challenges and issues surrounding the acceptance of genetically modified foods will need to be addressed before crop plants engineered for increased resistance to fungal pathogens are successfully brought to market (Hilder and Boulter 1999; Barton and Dracup 2000; Kaeppler 2000). These issues include: potential spread of the trait to closely related weedy species and impact on their ecology; possibility of nontarget effects on other diseases, pests, or beneficial microorganisms; potential health effects of the overexpression of antimicrobial proteins in foods; and possibility of promoting new pathogen strains with resistance to or that are able to overcome the novel engineered trait.

The spread of novel engineered genes from crop plants to weedy relatives has been demonstrated in some plant species through movement of pollen (Hails 2000; Wilkinson et al. 2000). Could a weedy sexually compatible relative of a crop species benefit from the potential introduction and expression of a disease-resistant phenotype? Weeds are known to harbour inoculum of a wide range of fungal pathogens (Agrios 1997) and these pathogens may maintain a balance over weed growth. The potentially accelerated growth of a weedy species via introgression of the disease-resistant transgene, however, may be balanced by the reduction in primary inoculum that can be generated from weedy hosts adjacent to commercial crop fields, thereby reducing disease pressure. The extent to which the fitness of a weedy species may be enhanced by introgression of a disease-resistant phenotype has yet to be evaluated.

Nontarget effects on other diseases, pests, or beneficial microorganisms will have to be monitored in crop plants engineered to express antifungal or antimicrobial compounds. While unpredicted beneficial effects against other related fungal pathogens may be a positive aspect, an assessment of the effects on unrelated fungi, viruses, or bacteria may need to be conducted. It is unwieldy for researchers involved in the development of genetically engineered plants to screen against a multitude of diseases or pathogens common to that crop, an approach that may be taken by plant breeders during development of a new cultivar. The results in Table 1 demonstrate the specificity of the evaluation approach used for transgenic plants, which is conducted mostly under axenic or controlled environment conditions, and which infrequently includes more than one pathogen for challenge inoculation. A report of enhanced resistance to tobacco mosaic virus and potato virus X in a β-1,3-glucanase-deficient tobacco mutant (Iglesias and Meins 2000) suggests that plants overexpressing this enzyme to enhance fungal resistance should be tested for potentially enhanced susceptibility to viruses. Antisense transformation of tobacco to produce β-1,3-glucanase-deficient plants showed that these plants had increased deposition of callose in response to infection and had fewer lesions due to tobacco mosaic virus and tobacco necrosis virus (Beffa et al. 1996). How would the overexpression of antimicrobial compounds in the roots of genetically engineered plants alter their compatibility with mycorrhizae or beneficial endophytic fungi, or with various rhizosphere-colonizing microbes that could inhibit the development of soilborne pathogens? Evaluations of these potential effects have been conducted in a few studies (Vierheilig et al. 1993, 1995; Lottmann et al. 2000; Lukow et al. 2000; Lottmann and Berg 2001). No side effects have been found so far, except for one study in which cultivar-specific alterations in rhizosphere bacteria were found in a transgenic canola line (Siciliano and Germida 1999). Joint collaborations between molecular biologists and plant pathologists should foster the appropriate evaluations of these transgenic plants.

The potential of antimicrobial compounds to act as allergens or toxins when consumed by humans (Franck-Oberaspach and Keller 1997) would require that the guidelines established by the appropriate regulatory agencies be followed for the countries where the crops are grown and marketed (Kaeppler 2000).

The possibility of selecting pathogen strains with resistance to the engineered trait may be increased with the widespread deployment of transgenic crops expressing specific antimicrobial compounds or that have broad-spectrum disease resistance. Fungal pathogens have demonstrated the capability for rapid change in genetic structure in the face

of selection forces, such as highly specific fungicides, major disease resistance genes, and environmental factors. The selection imposed by antimicrobial proteins, for example, could force the evolution of adaptive strategies in the pathogen to defend against the inhibitory compounds. Such a co-evolution has been proposed for chitinases (Bishop et al. 2000), in which adaptive functional modifications of the enzyme active site have occurred. Similarly, changes in sensitivity of pathogens to antimicrobial proteins overexpressed in transgenic plants could be selected. The use of combined genes that target different sites could reduce the selection pressure imposed on the pathogen. Genetically engineered plants with successfully enhanced disease resistance should not be viewed as a panacea and continual monitoring for unexpected events will be necessary.

Future prospects

The tremendous scientific progress made since 1991 in genetic engineering of plants for enhanced resistance to fungal pathogens as described in this paper is an indication of the high level of interest in the scientific community on this subject. As the technology evolves toward the use of tissue-specific or pathogen-inducible promoters, the expression of engineered traits that are effective against a broad range of pathogens, and the utilization of synthetically derived peptides and of R genes, the impact on disease management will be enhanced. Evaluation of these transgenic plants for response to disease will need to be extended to field trials and appropriate agronomic data collected to ensure that this technology can be successfully implemented in farmer's fields to augment on-going disease management practices. Transgenic plants with enhanced disease resistance can become a valuable component of a disease management program in the future.

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